

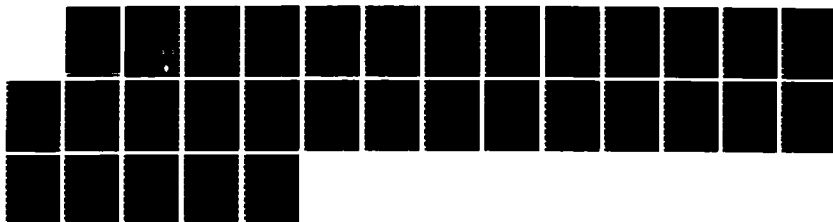
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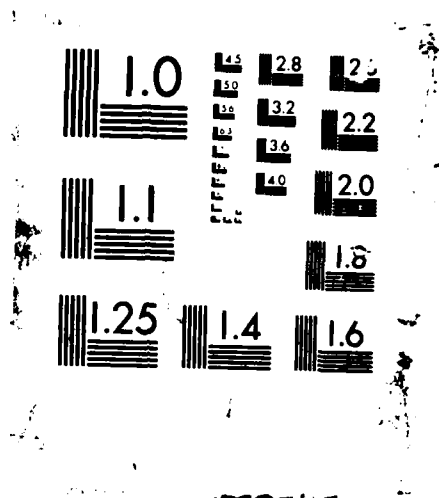
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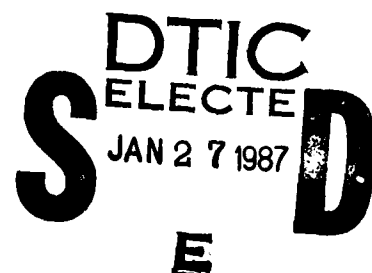
**LABORATORY TECHNIQUES FOR
DETERMINING THE EFFECTS OF
PYRIDOSTIGMINE BROMIDE**

**Faust R. Parker, Jr., Ph.D.
Judith A. Barber, B.A.
Estrella M. Forster, B.S.
James E. Whinnery, Ph.D., M.D. (USAFSAM/VNAEL)**

**Rothe Development, Incorporated
4614 Sinclair Road
San Antonio, Texas 78222**

November 1986

Interim Report for Period May 1985 - May 1986



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Prepared for

**USAF SCHOOL OF AEROSPACE MEDICINE
Aerospace Medical Division (AFSC)
Brooks Air Force Base, TX 78235-5301**



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NOTICES

This interim report was submitted by Rothe Development Incorporated, 4614 Sinclair Road, San Antonio, Texas 78222, under contract F33615-85-D-4510, job order 2729-11-A1, with the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, Brooks Air Force Base, Texas. Dr. James E. Whinnery (USAFSAM/VNAEL) was the Laboratory Project Scientist-in-Charge.

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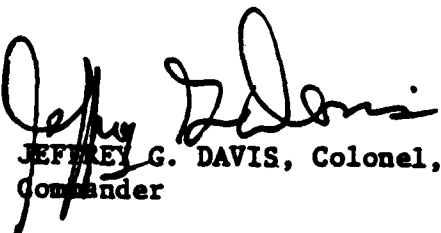
The voluntary informed consent of the subjects used in this research was obtained in accordance with AFR 169-3.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.


JAMES E. WHINNERY, PH.D., M.D.
Project Scientist


W. C. ALEXANDER, Ph.D.
Chief, Crew Technology Division


JEFFREY G. DAVIS, Colonel, USAF, MC
Commander

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LABORATORY TECHNIQUES FOR DETERMINING THE EFFECTS OF PYRIDOSTIGMINE BROMIDE

INTRODUCTION

Pyridostigmine bromide (PB), a reversible anticholinesterase pharmacologic agent, is currently in the U.S. Air Force (USAF) chemical defense armamentarium to protect aircrew and groundcrew members from the lethal effects of chemical warfare nerve agents, such as Soman. According to current USAF policy, PB, in the form of 30-mg tablets, will be given every 8 hr as a preexposure antidote. The use of this antidote is not without concern for the USAF aircrews of high performance fighter aircraft, since specific side effects could result from taking PB (16). The aeromedical research community has therefore been tasked with determining the tolerability of PB by subjects undergoing the stresses of flying combat missions. Of particular concern is whether or not PB affects: performance; tolerance for very rapid onset high sustained +G_z stress, or altitude stress; and spatial orientational abilities. In support of this clinical aeromedical research program, vital importance is attached to accurate laboratory confirmation of the levels of PB in the blood, and of the effect of PB on the inhibition of acetylcholinesterase (AChE). These laboratory analyses assist in safety assurance, confirm the reliability of volunteer subjects taking PB in double-blind clinical studies, and define the adequacy of dose-timing requirements of PB for providing optimal protection against chemical warfare nerve agents.

This report documents the establishment of reliable laboratory techniques for analysis of PB and AChE inhibition. PB analysis requires the use of high performance liquid chromatography (HPLC), and AChE inhibition determinations utilize spectrophotometric techniques. These analytical techniques are not routine, and require research-level procedures to establish their sensitivity and reproducibility. The main purpose of this report is to describe in detail the analytical techniques used at the USAF School of Aerospace Medicine (USAFSAM) to support the clinical research programs involving the physiological and performance aspects of taking PB. Laboratory results from human volunteers ingesting 30-mg and 60-mg tablets of PB are provided to demonstrate the reliability of current techniques. (For a review of the aeromedical aspects of PB ingestion, refer to Ref. 16.)

METHODS

Subject Selection

All subjects were volunteer subjects or aircrew who had been advised, prior to PB ingestion, of the known possible side effects of the drug. Subjects taking the 60-mg screening dose were under medical observation for idiosyncratic symptoms for 8 hr. Aircrew involved in the 30-mg study were

required to ingest a 30-mg tablet while they were on the ground, and remain under medical supervision for 4 hr before subsequent participation in single dose (30-mg) inflight studies.

Sample Collection and Storage

Blood samples were collected from subjects before ingestion of both 60- and 30-mg doses of PB. Post-PB-ingestion sampling occurred hourly for the 60-mg tablet, and at the end of flight operations for the 30-mg tablet. Samples were drawn from the antecubital vein directly into EDTA*-treated, silicone-coated, vacutainer tubes, and placed immediately on ice. One subject in the 60-mg study received an indwelling catheter with a heparin lock in lieu of an hourly venipuncture.

Iced samples were returned to the laboratory for immediate hematocrit and AChE activity determinations (8).** The remainder of each sample was stored as plasma at -70° C in silicone-treated, amber, screw-cap vials for subsequent HPLC analysis of PB levels.

Acetylcholinesterase Determinations

Equipment--The colorimetric enzyme reaction used in this assay was monitored with an LKB Ultraspectrophotometer, Model 4050 (LKB Instruments Inc., Gaithersburg, MD 20877). This unit was paired with an LKB Model 4070 Autofill Controller, equipped with a thermostated cuvette. The system was controlled with an Apple IIe (Apple Computer, Cupertino, CA 95014) computer using the LKB Enzyme Kinetics Software package.

Reagents--A Cholinesterase Reagent Set (#124117) from Boehringer Mannheim Diagnostics, Inc., was utilized for AChE activity determinations. Precilip, a lyophilized human serum normal lipid control (#125067; also from Boehringer Mannheim Diagnostics, Inc.), was used for daily control purposes. Blood samples were taken with lavender-stoppered Vacutainer (Becton Dickinson #6456) collection tubes. Millipore Milli-Q Reagent grade water was utilized for all procedures requiring deionized water.

Analytical Techniques--AChE activity was determined by using a colorimetric enzyme reaction, as developed by Ellman et al. (7) and utilized in the Boehringer Mannheim diagnostic kit. In this two-step reaction, acetylthiocholine is the substrate; and dithionitrobenzene (DTNB), the color reagent. Hydrolysis of the acetylthiocholine yields thiocholine, which reacts with DTNB to yield a yellow color. The increase in color intensity, directly proportional to AChE activity, was kinetically monitored at 405 nm and 25° C. Cholinesterase (ChE) activity was determined for whole blood and plasma, and AChE activity was calculated from these values.

* EDTA = ethylenediaminetetraacetic acid

** No sensitive (proprietary) material from the conference cited appears in this technical report.

Hematocrit determinations were initiated upon arrival of iced samples at the laboratory. Whole blood (0.200 ml) was pipetted into a 12 x 75 mm culture tube containing 1.8 ml of deionized water, vortexed for 3 sec, and then set aside to allow red cells to lyse. The remainder of the sample was centrifuged, and the plasma was separated by means of a plasma isolation filter.

The substrate (0.10 ml of 156 mM acetylthiocholine iodide) was added to 3 ml of a buffer/chromagen reagent (50 mM phosphate buffer, pH 7.2, and 0.25 mM (DTNB) in a culture tube. The appropriate whole blood lysate or plasma sample (0.02 ml) was added to this mixture, vortexed for 3 sec, and aspirated into the spectrophotometer cuvette. Each sample was tested for 3 min and in triplicate. A lyophilized human serum control was utilized before sample testing to establish that the assay would produce results within acceptable limits. A step-by-step outline of the spectrophotometer procedure is presented in Appendix C: Section 1.

Typical reaction-rate plots for the ChE determinations are shown in Appendix A (Fig. A-1). Formulas used for the determination of plasma and whole blood ChE and the calculations for red cell AChE are in Appendix B (items 1 and 2). From the difference in baseline AChE activity and post-PB ingestion AChE activity, the percent inhibition in activity can be expressed as percent inhibition. Examples of the final calculation procedure, from the results in items 1 and 2 (Appendix B), are in item 3 (Appendix B).

Pyridostigmine Assay

Instrumentation--The HPLC determinations of PB utilized a Waters Associates Model 721 System Controller, 730 Data Module, 510 Pump, 481 Variable Wavelength UV Detector, TCM Column heater, and a 710B Sample Processor (Waters Associates, Milford MA 07157). The detector wavelength employed was 208 nm at a sensitivity setting of 0.002 absorbance units full scale (AUFS). At a flow rate of 1.0 ml/min, separations were accomplished on an Altex Ultrasphere-octyl Column (4.6 mm x 25 cm, 5 μ m; Beckman Instruments, Inc., Berkeley CA 94710) using a mobile phase consisting of acetonitrile and water (30:70), 0.1% sodium lauryl sulfate (SDS), 0.1% H_2PO_3 , and 0.0025 M tetramethylammonium chloride (TMA^+Cl^-).

Reagents--Acetonitrile (CH_3CN) and phosphoric acid (H_3PO_4) [both HPLC grade] were purchased from Fisher Scientific (Houston, TX 77001), while SDS (99% purity) and TMA^+Cl^- (98% purity) were from Fluka Chemical Company (Hauppauge, NY 11787). PB was furnished by Roche Laboratories (Nutley, NJ 07110), and neostigmine bromide was obtained from Sigma Chemical Co. (St. Louis, MO 63178). C-2 bond elute cartridges (500 mg) were purchased from Analytichem International Inc. (Harbor City, CA 90710). Water was produced by a Milli-Q Reagent Water system from Millipore Corp. (Bedford, MA 01730). The SDS had to be recrystallized twice by dissolving in methanol, freezing overnight, vacuum filtering through a Type FH 5- μ m Millipore filter and, finally, drying in an oven.

Sample Preparation--Plasma samples (0.5 ml) were precipitated with 1 ml of CH₃CN containing the internal standard neostigmine bromide (40 ng/ml). After undergoing vortexing for 10 sec and centrifugation at 4,000 rpm for 10 min, the supernatant was poured onto a C-2 bond elute cartridge and the protein pellet was discarded. Cartridges were presaturated with 100% CH₃CN before loading, and extractions allowed isolation of pyridostigmine by gravity. The loaded sample was washed first with 2 ml of water, and next with 2 ml of 100% CH₃CN to eliminate undesirable endogenous substances. The bond elute cartridge was then washed with 1 ml of 95% CH₃CN, containing 0.05% SDS (twice recrystallized) and 0.05% TMA⁺Cl⁻; the final elution was with 3 ml of 95% CH₃CN, containing 0.1% SDS and 0.05% TMA⁺Cl⁻. This final fraction, containing the pyridostigmine and internal standard, was evaporated to dryness under N₂, reconstituted in 200 µl of 30% CH₃CN in water, and injected (175 µl) onto the column via the auto sampler. A step-by-step description of the HPLC procedure is given in Appendix C: Section 2.

Chromatography--A linear relationship exists between pyridostigmine plasma concentration and peak height ratio of the drug over internal standard. Linear regressions of peak height ratio vs. concentration were run prior to sample injections on each new batch of mobile phase. Regressions on standards of 50, 30, 16, 10, 6, 4, 2, and 0 ng/ml gave typical correlation coefficients (r²) of 0.9986, with a slope of 0.0220 and a Y intercept of 0.0008 (Appendix B: Table B-1).

Shown in Figure A-2 (Appendix A) are typical chromatographic tracings for plasma samples spiked with PB at 50, 16, 4, and 0 ng/ml. Pyridostigmine typically elutes at around 20 min; and the internal standard, neostigmine, at 30 min. These times varied slightly, depending on column condition, etc. We found that, for obtaining consistent chromatographs and maintaining column resolution, the best procedure involved leaving the mobile phase on overnight at a reduced flow rate of 0.2 ml/min, and flushing the system over the week-ends with acetonitrile and water (50:50) at the same flow rate.

Recovery was determined by comparing the difference between known amounts of pyridostigmine spiked in plasma and in 100% water. Each sample was prepared as previously described, except that the internal standard was not added until the N₂ evaporation stage and the 100% water spiked sample did not pass through the C-2 cartridge. Percent recovery was determined for each paired sample by the following formula:

$$\% \text{ recovery} = \frac{\text{peak height ratio PB in plasma}}{\text{peak height ratio PB in H}_2\text{O}} \times 100$$

Recoveries ranged from 64% to 77% (Appendix B: Table B-2).

The accuracy of the method for determining PB concentrations was established by the analysis of blind spiked plasma samples from within this laboratory. Spiked levels and measured levels are presented in Appendix B: Table B-3.

RESULTS

Shown in Figure 1 are the hourly levels of plasma PB and resultant percent AChE inhibition for two subjects (A and B), each ingesting a single 60-mg dose of PB.

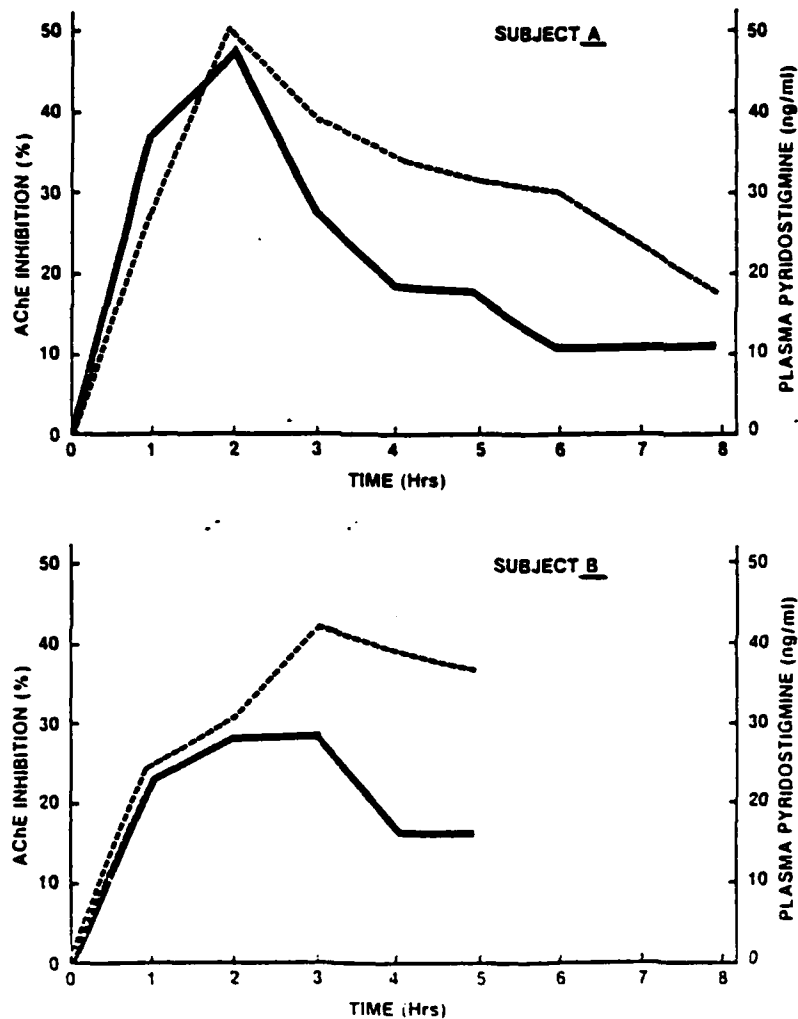


Figure 1: Subjects A and B. Plasma pyridostigmine (solid line) and AChE inhibition (dashed line) levels for subjects ingesting 60 mg of pyridostigmine bromide.

Given in Table 1 are the results from postflight samples on subjects ingesting a single 30-mg dose of PB. Two of these subjects also had samples drawn after missions were aborted post drug ingestion, so that no flight stress was incurred.

TABLE 1. PERCENT RBC AChE INHIBITION AND PLASMA PYRIDOSTIGMINE LEVELS FOR AIRCREW INGESTING 30-MG TABLETS OF PYRIDOSTIGMINE BROMIDE

Subject number	Time from ingestion (hr)	Weight (kg)	RBC ^a AChE ^b inhibition (%)	Plasma pyridostigmine (ng/ml)
1	3.2	68	23	10
2	3.0	93	24	18
3	4.2	82	31	21
4	2.8	71	20	11
5	4.2	78	31	10
6	3.1	81	16	11
7	3.0	73	5	10
8	3.1	68	28	18
9	2.3	71	25	14
10	3.3	84	17	10
11	3.0	73	18	15
12	3.4	64	17	14
13	3.3	75	27	17
14	3.4	84	0	11
15	2.0	75	2	14
16 ^c	2.0	75	24	11
16	3.0	75	7	8
17 ^c	2.0	80	49	29
17	3.8	80	31	25

^aRBC = red blood cell

^bAChE = acetylcholinesterase

^cSubject did not fly after drug ingestion.

DISCUSSION

Many methods have been reported for the assay of AChE activity, including: radiometry (9); fluorometry (15); electrometry (5); high performance liquid chromatography, using electrochemical detection (11,14); and colorimetry (7,12). Colorimetric methods have been widely accepted as routine enzyme assays for some time, and well suit the experimental design herein.

Other methods may be more precise for specific AChE tissue assays, especially where very small quantities are concerned; however, the preparation times and complexity of the assays do not lend themselves to the timely results which are of paramount importance to our operational and research studies. The turn-around time on AChE levels is also a prime concern; for the appropriate human-use committees have mandated that, in screening volunteer subjects with PB, none who exhibit more than 40% inhibition with 30-mg PB liquid will be allowed to participate in the studies. The method of Ellman et al. (7), in kit form by Boehringer Mannheim, is a convenient and consistent product which permits rapid assays in the range of precision required for nonpharmacological studies.

Liquid-liquid extractions of pyridostigmine, as described by Chan et al. (3), are tedious and give marginal results in the lower observed range (<10 ng/ml) of PB in plasma. The methods of Ellin et al. (6), more recently used by Breyer-Pfaff et al. (2), appear much simpler and more sensitive than those previously described; however, they have not been attempted in this laboratory. The solid-liquid extraction, described by Lin et al. (13)* and utilized herein, provides a simple, accurate, reproducible method for determining low levels of PB in plasma. The method appears to be at least as good as alternative procedures.

Comprehensive proof of the method was not completed in this laboratory, because Lin et al. (13)* had previously compiled this information. Those components (linearity, recovery, and accuracy) which were investigated, however, appear to compare quite favorably with Lin's data.

Data from two subjects, ingesting 60-mg tablets of PB, follow previously described single dose profiles for plasma pyridostigmine levels, with plasma PB peaks occurring at about 2 hr (1,4). The obvious correlation between PB plasma levels and AChE inhibition agrees with the findings of Kaminskis et al. (10)*, and the peak percent in AChE inhibition approximates that in plasma PB in both cases.

Results from 30-mg inflight studies gave AChE inhibition and PB plasma levels in the range expected. These data do not lend themselves to detailed analysis, due to low capability for variable control. Among the factors which could not be rigidly controlled were differential flight stress, aircrew

*No sensitive (proprietary) material from the conference cited appears in this technical report.

nutritional status, time from drug ingestion to blood draw, etc. Fair linear correlation ($r^2 = 0.706$) existed, however, between plasma PB levels and percent AChE inhibition (Appendix A: Figure A-3).

This study afforded an excellent opportunity for testing the 30-mg PB dose in an operational setting. In addition, the research provided a critically needed real-world examination of assay procedures for AChE and PB on which to base future studies.

CONCLUSION

The analytical techniques described herein are reliable methods for rapid determination of plasma PB and acetylcholinesterase inhibition resulting from oral ingestion of PB. These techniques will be used at USAFSAM in all future studies that are designed to evaluate the aeromedical aspects of PB and its potential effects on aviation-related performance and stress tolerance.

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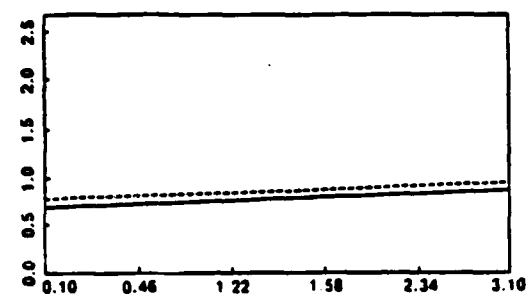
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.. APPENDIX A:

Figures A-1, A-2, and A-3

--APPENDIX A--

A. WHOLE BLOOD BASELINE



TITLE : ACH/TM/BSLN/LYSAT

IDENTIFICATION : (SUBJECT A)

LAG TIME : 0 MIN 10 SEC

DURATION : 3 MIN 0 SEC

MINIMUM ABS : 0

MAXIMUM ABS : 2.5

FACTOR : 117000

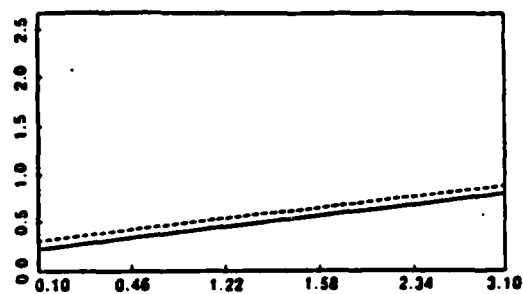
WAVELENGTH : 405

SAMPLE NUMBER : 1

ABSORBANCE : INITIAL 0.714 FINAL 0.875 CHANGE 0.160

RATE OF CHANGE 0.053 A/MIN ACTIVITY 6223 IU STD DEV 0.002

B. PLASMA BASELINE



TITLE : ACH/TM/BSLN/PLASMA

IDENTIFICATION : (SUBJECT A)

LAG TIME : 0 MIN 10 SEC

DURATION : 3 MIN 0 SEC

MINIMUM ABS : 0

MAXIMUM ABS : 2.5

FACTOR : 11700

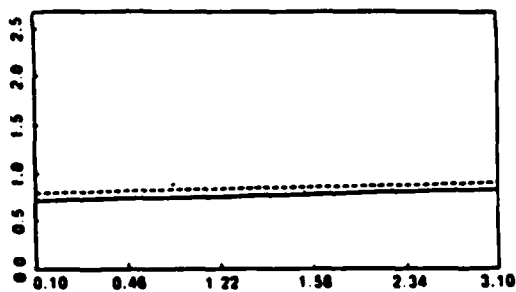
WAVELENGTH : 405

SAMPLE NUMBER : 1

ABSORBANCE : INITIAL 0.235 FINAL 0.825 CHANGE 0.590

RATE OF CHANGE 0.196 A/MIN ACTIVITY 2298 IU STD DEV 0.003

C. WHOLE BLOOD 60mg PB AT 3 HR.



TITLE : ACH/TM/60mg/3HR/LYSAT

IDENTIFICATION : (SUBJECT A)

LAG TIME : 0 MIN 10 SEC

DURATION : 3 MIN 0 SEC

MINIMUM ABS : 0

MAXIMUM ABS : 2.5

FACTOR : 117000

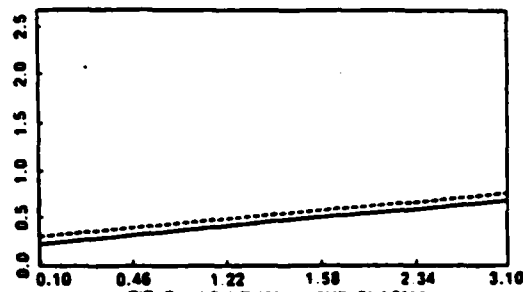
WAVELENGTH : 405

SAMPLE NUMBER : 3

ABSORBANCE : INITIAL 0.719 FINAL 0.824 CHANGE 0.104

RATE OF CHANGE 0.036 A/MIN ACTIVITY 4092 IU STD DEV 0.002

D. PLASMA 60mg PB AT 3 HR.



TITLE : ACH/TM/60mg/3HR/PLASMA

IDENTIFICATION : (SUBJECT A)

LAG TIME : 0 MIN 10 SEC

DURATION : 3 MIN 0 SEC

MINIMUM ABS : 0

MAXIMUM ABS : 2.5

FACTOR : 11700

WAVELENGTH : 405

SAMPLE NUMBER : 3

ABSORBANCE : INITIAL 0.215 FINAL 0.673 CHANGE 0.459

RATE OF CHANGE 0.152 A/MIN ACTIVITY 1773 IU STD DEV 0.002

Figure A-1. Typical spectrophotometric cholinesterase assay printouts of whole blood (A and C), and plasma (B and D) from a 60-mg PB test subject.

--APPENDIX A--

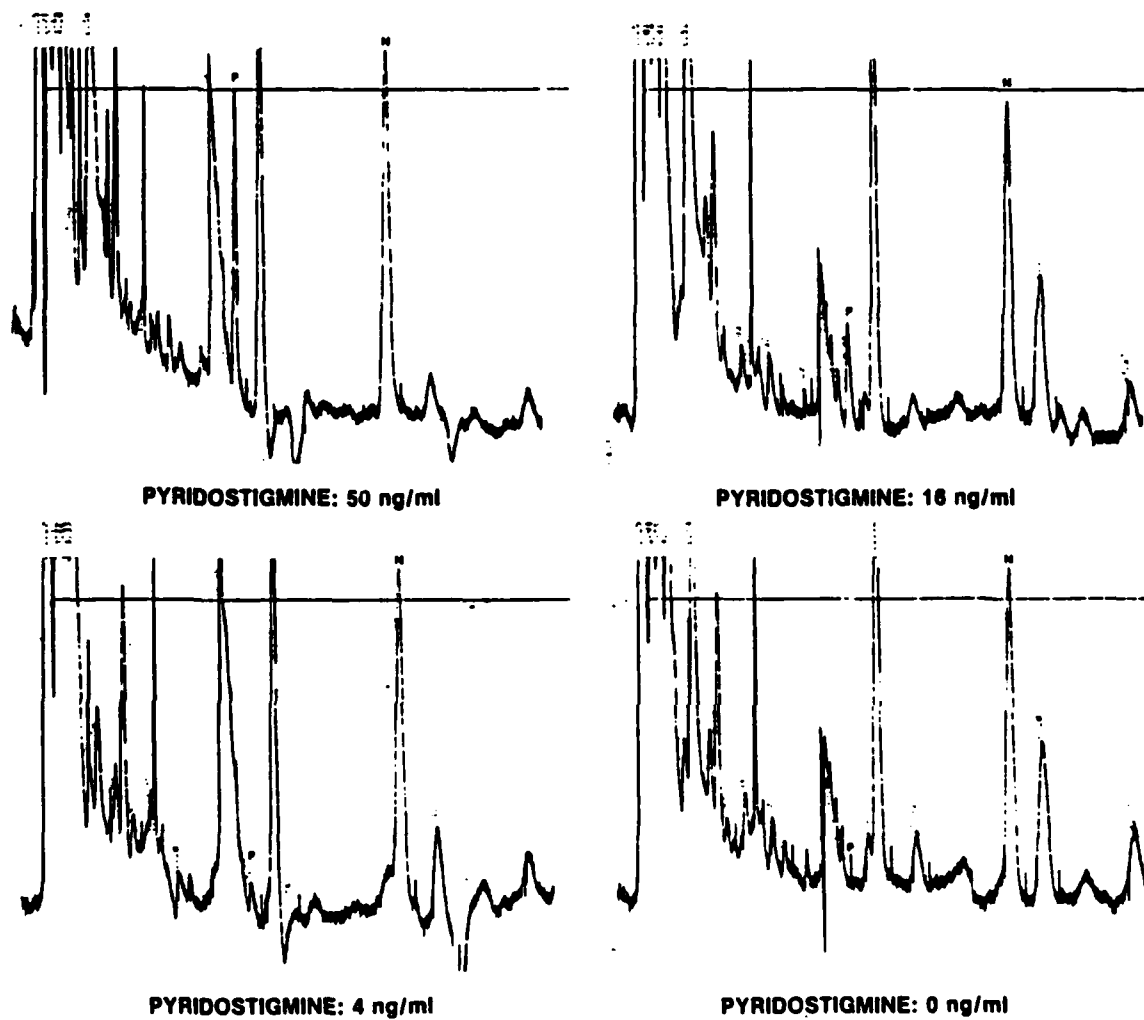


Figure A-2. Typical chromatographs for plasma spike pyridostigmine bromide at 50, 16, 4, and 0 ng/ml and 40 ng/ml of the internal standard.

--APPENDIX A--

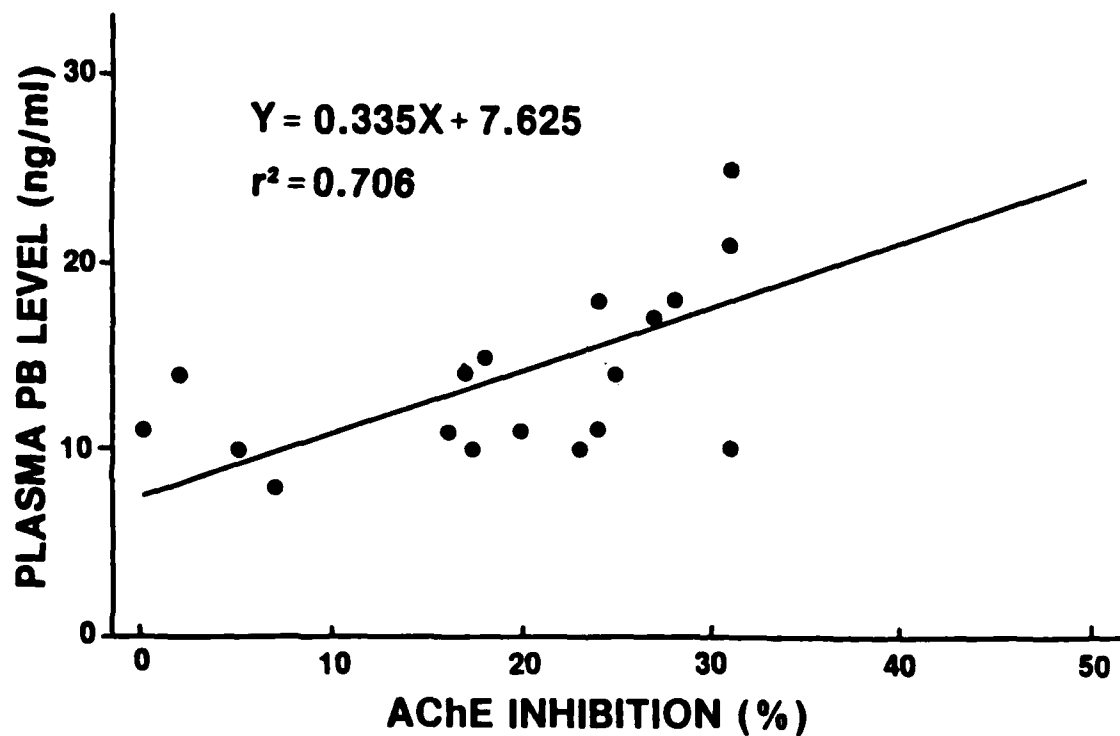


Figure A-3. Correlation of circulating pyridostigmine bromide levels and percent AChE inhibition in postflight aircrew subjects.

APPENDIX B:

Items 1, 2, and 3

and

Tables B-1, B-2, and B-3

APPENDIX B

1. CALCULATION OF PLASMA AND WHOLE BLOOD LYSAT CHOLINESTERASE

Activity levels of ChE are expressed in international enzyme units per liter (U/l), defined as the activity of enzyme that converts 2 μ mole/l of substrate in 1 min at standard conditions.

The formula used for the determination of plasma ChE activity in the BMC kit is:

$$\frac{\Delta A/\text{min} \times \text{total assay volume (ml)} \times 1000}{\text{abs. coeff.} \times \text{light path (cm)} \times \text{spec. vol. (ml)}} = \text{mU/ml specimen}$$

where: Δ = change

A = absorbance

1000 = factor for conversion of units/ml to milliunits/ml

abs. coeff = absorbandy coefficient = 13.3 cm/ μ mole
dithiobenzoic acid at 405 nm

With a light path of 1 cm, the formula then becomes:

$$\text{at 405 nm: } \frac{\Delta A/\text{min} \times 3.12 \times 1000}{13.3 \times 1 \text{ cm} \times 0.02 \text{ ml}} = A/\text{min} \times 11700^* = \text{mU/ml specimen}$$

*The whole blood ChE calculation follows that of the plasma with one exception: The preparation of the whole blood lysat constitutes a 10-fold dilution which, by applying the same formula, would change the plasma factor by 10, thus making the factor for calculating the ChE activity of the whole blood lysat 117000.

2. CALCULATION OF ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase, found on the envelope of the red blood cell, is difficult to quantify directly. With the kit from Boehringer Mannheim Diagnostics, Inc., the RBC AChE activity is a calculated value. The calculation is as follows:

$$\text{RBC} = \frac{\text{WB} - [\text{Pl} \times (1 - \text{Hct})]}{\text{Hct}}$$

where: RBC = red blood cell activity,

WB = whole blood activity,

Pl = plasma activity, and

Hct = hematocrit (expressed as a decimal equivalent).

--APPENDIX B--

Using the whole blood lysat and plasma baseline values in Appendix A (Fig. A-1), and plugging them into the foregoing formula, the RBC AChE activity becomes:

$$\text{RBC} = \frac{6223 - [2298 \times (1 - 0.46)]}{0.46}$$

$$\text{RBC} = \frac{6223 - 1241}{0.46}$$

$$\text{RBC} = 10,830 \text{ U/l.}$$

3. FORMULA FOR CALCULATING PERCENT INHIBITION AND SAMPLE CALCULATION

$$\begin{aligned} \% \text{ Inhibition} &= \left(1.00 - \frac{\text{AChE activity 60 mg PB dose at 3 hr}}{\text{AChE activity baseline}} \right) \times 100 \\ &= \left(1.00 - \frac{6,815}{10,830} \right) \times 100 \\ &= (1.00 - 0.63) \times 100 \\ &= 0.37 \times 100 \\ &= 37\% \end{aligned}$$

--APPENDIX B--

TABLE B-1. PLASMA PYRIDOSTIGMINE PEAK HEIGHT RATIO

Concentration (ng/ml)	Observed peak height ratio	Expected peak height ratio
50	1.107	1.101
30	0.655	0.661
16	0.352	0.353
10	0.176	0.221
6	0.139	0.133
4	0.112	0.089
2	0.056	0.045
0	0.000	0.001

Regression equation: $Y = 0.0220X + 0.0008$
 $r^2 = 0.9986$

TABLE B-2. RECOVERY OF PYRIDOSTIGMINE BROMIDE IN PLASMA

Concentration (ng/ml)	Percent recovery ^a	Peak height ratio	
		Water sample	Plasma sample
80	64	1.729	1.108
40	70	0.789	0.550
16	69	0.453	0.311
6	77	0.208	0.160

^aPercent recovery based on the formula:

$$\% \text{ Recovery} = \frac{\text{Peak height ratio plasma sample}}{\text{Peak height ratio water sample}} \times 100$$

--APPENDIX B--

TABLE B-3. ACCURACY OF ANALYTICAL METHOD FOR DETERMINATIONS OF
PYRIDOSTIGMINE BROMIDE IN PLASMA

Spiked level (ng/ml)	Measured levels (ng/ml)		Statistical data
4.0	4.3	4.2	N = 11
	4.1	3.8	Mean = 4.05
	4.0	4.1	Std dev = 0.28
	3.9	3.7	Coef var % = 6.60
	4.3	3.6	Bias = 1.2%
	4.5		
18.0	16.9	16.6	N = 11
	19.0	19.4	Mean = 17.12
	15.7	16.4	Std dev = 1.13
	16.9	17.1	Coef var % = 6.60
	17.4	16.2	Bias = 4.9%
	16.7		
46.0	43.1	45.4	N = 11
	39.8	39.9	Mean = 43.04
	37.2	44.4	Std dev = 3.30
	46.4	43.9	Coef var % = 7.67
	40.1	45.6	Bias = 6.4%
	47.5		

APPENDIX C:

Sections 1 and 2

APPENDIX C

SECTION 1: AChE INHIBITION SPECTROPHOTOMETRY

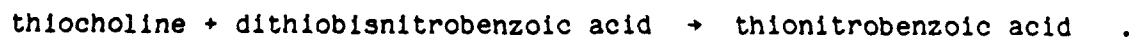
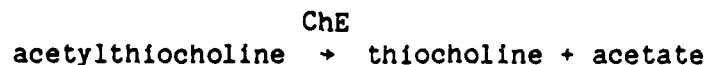
A. Equipment:

1. LKB Ultrospectrophotometer, Model 4050
2. LKB Autofill Controller with Thermostated Cuvette, Model 4070
3. Apple IIe computer
4. Epson printer
5. LKB Enzyme Kinetics Software Package

B. Assay Procedure:

1. Blood is collected, in EDTA-treated vacutainer tube, from the antecubital vein of human subjects and immediately placed on ice.
2. Hematocrit value is determined.
3. Whole blood lysat preparation: 1.8 ml water + 0.200 ml whole blood.
4. Remainder of blood sample is centrifuged to obtain plasma.
5. Test procedure--pipette into test tube:
 - a. 3.00 ml chromagen/buffer (50.00 mM phosphate buffer, pH 7.2) (0.25 mM dithiobisnitrobenzoic acid).
 - b. 0.10 ml substrate (156 mM acetylthiocholine iodide).
 - c. 0.02 ml whole blood lysat or plasma.

6. Chemical reactions:



Acetate and thiocholine react with dithiobisnitrobenzoic acid to form the yellow colored 2-nitro-5-mercapto benzoate that results in an increasing color intensity that is directly proportional to cholinesterase activity.

--APPENDIX C--
(Section 1)

7. All tests are conducted at 25° C.

8. The rate of change of absorbance/minute is recorded at 405 nm over a 3-min period.

9. Results are given in international enzyme units (U/l), defined as the activity of enzyme which converts 1 μ mole/l of substrate in 1 min at standard conditions.

10. Erythrocyte acetylcholinesterase (RBC AChE) activity is calculated, using plasma and whole blood lysat activity and sample hematocrit, from the following formula:

$$\text{RBC AChE} = \frac{\text{WB} - [\text{Pl} \times (1 - \text{Hct})]}{\text{Hct}}$$

in which: WB = cholinesterase activity of whole blood lysat,
Pl = cholinesterase activity of plasma, and
Hct = hematocrit expressed as a decimal equivalent.

--APPENDIX C--

SECTION 2: PYRIDOSTIGMINE BROMIDE CHROMATOGRAPHY

A. Equipment:

1. Waters Associates Model 721 System Controller
2. Waters Associates Model 730 Data Module
3. Waters Associates Model 510 Pump
4. Waters Associates Model 481 UV Detector
5. Waters Associates Model TCM Column Heater
6. Waters Associates Model 710 B Sample Processor
7. Altex Ultra-Sphere C-8 Octyle Column (4.6 mm x 24 cm, 5 μ m)
8. Analytichem International C-2 Bond Elute Cartridge (500 mg).

B. Chemical Preparation - Chromatography:

1. Mobile Phase (4 liters)

a. To 2,800 ml of Milli-Q grade water, add 4 g SDS (sodium lauryl sulfate), 1.656 g TMA^+Cl^- , and 4 ml phosphoric acid. Place on magnetic stirrer until completely dissolved.

b. Filter under vacuum, using a Millipore HATF 0.45 μ m filter.

c. Filter 1,200 ml acetonitrile, using vacuum and a Millipore FHUP 0.5 μ m filter.

2. SDS recrystallization

a. To 100 g of SDS, add two volumes of HPLC grade methanol. Place on magnetic stirrer until all SDS has dissolved into a slurry (several hours).

b. Place in a freezer overnight. Then filter under vacuum through a Millipore HATF 0.45 μ m filter. Redissolve SDS in two more volumes of methanol as before; freeze; and filter again.

--APPENDIX C--
(Section 2)

c. After the second filtering, place SDS in a large beaker and put in a drying oven overnight. Stir several times during the next day, and be sure that the SDS has a flaky, talc-like appearance. If not, continue recrystallization procedure.

3. Standard solutions

a. Pyridostigmine bromide (PB): Place 10 mg of PB in a volumetric flask and bring volume to 100 ml with Milli-Q grade water. Add 1 drop of concentrated hydrochloric acid. Keep refrigerated.

b. Neostigmine bromide: Place 10 mg of neostigmine bromide in a volumetric flask and bring volume to 100 ml with Milli-Q grade water. Add 1 drop of concentrated hydrochloric acid. Keep refrigerated.

4. Working solutions

a. Pyridostigmine bromide: Place 1 ml of PB standard solution in a volumetric flask and bring volume to 100 ml with Milli-Q grade water. Keep refrigerated. (1 μ l = 1 ng)

b. Neostigmine bromide: Place 1 ml of neostigmine bromide standard solution in a volumetric flask and bring volume to 100 ml with Milli-Q grade water. Keep refrigerated. (1 μ l = 1 ng)

c. Neostigmine internal solution: Place 40 μ l of the standard neostigmine bromide solution in a volumetric flask and bring volume to 100 ml with filtered acetonitrile. Keep refrigerated. (1 ml = 40 ng of internal standard)

5. Elute solutions (500 ml)

a. Elute solution (0.50% SDS, 0.50% TMA⁺Cl⁻): 475 ml of filtered acetonitrile is added to 0.250 g of recrystallized SDS and 0.250 g of TMA⁺Cl⁻ in a glass-stoppered reagent bottle. Then 25 ml of Milli-Q grade water is added, and the solution is placed on a magnetic stirrer until dissolved.

b. Elute solution (0.100% SDS, 0.05 TMA⁺CL⁻): Prepare as in 5-a; but use 0.500 g of recrystallized SDS instead of 0.250 g.

--APPENDIX C--
(Section 2)

C. Elution Procedure:

1. Pre-saturate C-2 Bond Elute Cartridges with CH_3CN .
2. To 0.5 ml plasma in a 12 x 75 mm disposable culture tube, add 1 ml of CH_3CN for control (non-PB) tubes or 1 ml of CH_3CN - neostigmine internal standard solution to precipitate the plasma proteins. Each tube is labeled using 3/4-in labeling tape, which is later used to hold the C-2 cartridge in place.
3. Vortex for 10 sec, and centrifuge at 3500 rpm for 10 min.
4. Using the label from each 12 x 75 mm culture tube, tape the appropriate C-2 cartridge in the top of a 13 x 100 mm disposable culture tube and pour the liquid portion of the sample onto the cartridge. The protein pellet will remain in the bottom of the tube and is discarded. Allow sample to gravity-drip through the cartridge.
5. Wash cartridge with 2 ml of Milli-Q water, and then with 2 ml of CH_3CN to remove undesirable endogenous substances.
6. Wash with 1 ml of 0.05% SDS - 0.50% TMA^+Cl^- elute solution.
7. Tape the C-2 cartridge to the top of a new 12 x 75 mm disposable culture tube (discard contents of the 13 x 100 mm tube), and elute with 3 ml of 0.1% SDS - 0.05% TMA^+Cl^- elute solution.
8. Evaporate the final elute under ultra pure nitrogen to dryness. A fine white residue will be in the bottom of the tube.
9. Reconstitute with 200 μl of 30% CH_3CN -Milli-Q water, and vortex until all residue has dissolved. Load into limited volume inserts and place in a Wisp Carousel.
10. After using C-2 cartridges, wash with one volume of the 0.1% SDS - 0.05% TMA^+Cl^- solution prior to flushing with CH_3CN .

D. Chromatographic Assay Conditions

1. Column: Altex Ultrasphere C-8 Octyl column (4.6 mm x 25 cm, 5 μm) maintained at 28° C.
2. Flow rate: 1.0 ml/min
3. Wavelength: 208 nm
Detector setting: sensitivity = 0.002 AUFS

--APPENDIX C--
(Section 2)

4. Data module parameters:
 - a. Peak width - 25
 - b. Noise rejection - 10
 - c. Chart speed - 0.5 cm/min
5. Wisp parameters:
 - a. Run time: 45 min
 - b. Injection volume: 175 μ l.
6. Retention times: In the range of 18-21 min for pyridostigmine bromide and 29-32 min for neostigmine bromide, depending on column performance.

END

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